

RAPID COMMUNICATION

Genetic Characterization of the Pathogenic Influenza A/Goose/Guangdong/1/96 (H5N1) Virus: Similarity of Its Hemagglutinin Gene to Those of H5N1 Viruses from the 1997 Outbreaks in Hong Kong

Xiyun Xu,^{*1} Kanta Subbarao,* Nancy J. Cox,* and Yuanji Guot

**Influenza Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333; and †Chinese National Influenza Center, Institute of Virology, Chinese Academy of Preventive Medicine, Beijing, China 100052*

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Analysis of the sequences of all eight RNA segments of the influenza A/Goose/Guangdong/1/96 (H5N1) virus, isolated from a sick goose during an outbreak in Guangdong Province, China, in 1996, revealed that the hemagglutinin (HA) gene of the virus was genetically similar to those of the H5N1 viruses isolated in Hong Kong in 1997. However, the remaining genes showed greater similarity to other avian influenza viruses. Notably, the neuraminidase gene did not have the 19-amino-acid deletion in the stalk region seen in the H5N1 Hong Kong viruses and the NS gene belonged to allele B, while that of the H5N1 Hong Kong viruses belonged to allele A. These data suggest that the H5N1 viruses isolated from the Hong Kong outbreaks derived their HA genes from a virus similar to the A/Goose/Guangdong/1/96 virus or shared a progenitor with this goose pathogen. © 1999 Academic Press

An influenza A virus that is avirulent can become virulent by the acquisition of genetic features, such as multibasic cleavage sites or glycosylation sites in the hemagglutinin (HA) gene, as was seen in the outbreaks of disease in chickens in Pennsylvania in 1983 and in Mexico in 1994 (1, 2). A virus that could previously infect only one species can infect a broader range of hosts or become pathogenic to other hosts if, by reassortment, it acquires a gene constellation that confers a broader host range or that specifies virulence. Reassortment is the mechanism by which the influenza A viruses that caused the 1957 and 1968 pandemics in humans are thought to have arisen (3).

In 1997, an avian influenza A (H5N1) virus infected 18 hospitalized patients in the Hong Kong Special Administrative Region, China; 6 of the patients died (4). A simultaneous outbreak of disease occurred in chickens in Hong Kong (5). The viruses that caused the human and chicken outbreaks were genetically and antigenically closely related (5–7). Currently, no explanation is available for the infectivity and pathogenicity of this avian virus for humans. Moreover, unlike the case of the 1983 outbreak of highly pathogenic avian influenza in chickens in Pennsylvania (2), no avirulent precursor virus for the virulent H5N1 viruses has been detected in Hong

Kong. No new H5N1 viruses were isolated from chickens since the live bird markets in Hong Kong were closed and the birds were slaughtered (5). When import of chickens into Hong Kong resumed, they were sold in different markets than were waterfowl, and surveillance in both types of markets was continued. The recent isolation of an influenza A (H5N1) virus from a goose in a Hong Kong market highlights the importance of this surveillance effort and the continuing potential threat posed by H5N1 influenza A viruses infecting waterfowl (8).

Highly pathogenic avian influenza viruses bearing an H5 HA were first isolated in shorebirds (A/Tern/South Africa/61) and have since been isolated from poultry for more than 3 decades, most notably from a limited outbreak in turkeys in Ontario, Canada, in 1966, chickens in Pennsylvania in 1983–1984, turkeys in Norfolk, England, in 1991–1992, and chickens in Mexico in 1994–1995 (9–11). Outbreaks of disease due to highly pathogenic avian influenza viruses of the H5 subtype in geese were not reported until 1996. During the summer and early fall of 1996, an outbreak of disease with 40% morbidity occurred on a goose farm in Guangdong Province, China. At least two influenza A (H5N1) viruses from sick birds were isolated in the allantoic cavities of 9- to 11-day-old embryonated eggs. Viral hemagglutinin (HA) and neuraminidase (NA) subtypes were determined at the Chinese National Influenza Center in Beijing, China, with a panel of antisera to different subtypes of influenza A

¹ To whom correspondence and reprint requests should be addressed. Fax: (404) 639-2334. E-mail: xxx1@cdc.gov.

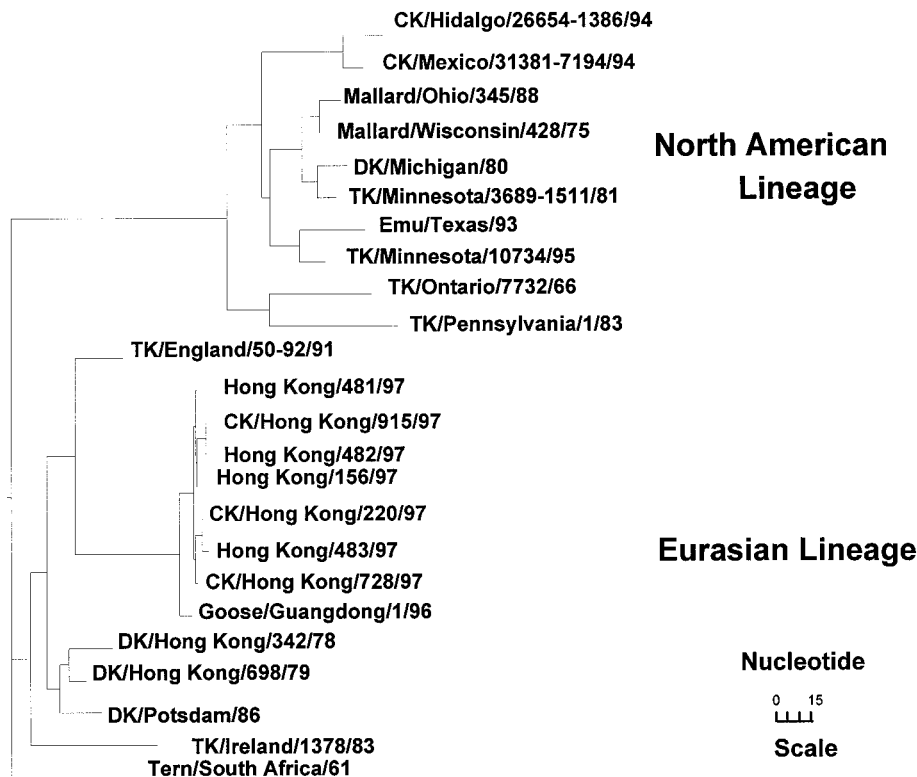


FIG. 1. Phylogenetic relationships of the HA1 domain of the hemagglutinin gene of H5 viruses and the *A/Goose/Guangdong/1/96* virus. All of the H5 subtype HA sequences available in GenBank were included. Version 3.5 of the Phylogeny Inference Package was used to estimate phylogenies from the nucleotide sequences. The tree was generated using neighbor-joining analysis determined by PHLYIP and is rooted to the *A/Tern/South Africa/61* virus. Horizontal distances are proportional to the number of nucleotide differences between branch points.

viruses (antisera were kindly provided by Dr. Robert Webster, at St. Jude Children's Research Hospital, Memphis, TN). The pathogenicity of one of these isolates, *A/Goose/Guangdong/1/96*, was evaluated in experimentally inoculated geese and the virus caused illness and death (Y. Guo, personal communication). This virus has also been reported to cause illness and death in chickens experimentally inoculated by the intravenous route (12).

To study the genetic properties of the H5N1 viruses causing this outbreak, and to better understand the genetic relationships between the H5N1 viruses isolated from geese in south China and from chickens and humans in Hong Kong in 1997, all eight gene segments of the influenza *A/Goose/Guangdong/1/96* virus were sequenced and compared phylogenetically with sequences available in GenBank and those of the influenza A H5N1 isolates from Hong Kong. Influenza *A/Goose/Guangdong/1/96* virion RNA was extracted using the RNeasy RNA extraction Kit (Qiagen) at the Chinese National Influenza Center in Beijing, China. cDNA synthesis and PCR amplification of the coding region of the viral gene segments were carried out in one or two parts, using gene-specific primer sets (sequences of the primers available on request) at the Centers for Disease Control and Prevention. PCR-derived dsDNA was used

as a template for sequencing on an Applied Biosystem 373A automated DNA sequencer using cycle sequencing dye terminator chemistry (Perkin-Elmer, Foster City, CA). RT-PCR and sequence analysis of the *A/Goose/Guangdong/1/96* virus and the *A/Hong Kong/156/97* virus were carried out during different weeks in the laboratory. Negative controls were included and remained negative in all PCR experiments. RT-PCR and sequencing of the HA gene of the *A/Goose/Guangdong/1/96* virus was repeated to confirm the results and the sequence data obtained on the two occasions were identical. The sequences of the primers used for these reactions are also available upon request. The nucleotide sequences were analyzed using version 8.0 of the sequence analysis software package of the University of Wisconsin at Madison, Genetic Computer Group. GenBank Accession numbers for the sequences obtained from this study are AF144300 to AF144307.

The entire coding region of the HA gene of the *A/Goose/Guangdong/1/96* virus was sequenced. Multiple basic amino acids (RERRRKKR) were observed at the cleavage site between the HA1 and HA2 domains of the *A/Goose/Guangdong/1/96* virus; this sequence at the cleavage site was identical to that of the H5 viruses isolated from Hong Kong (13, 14). The motif of multiple basic amino acids at the cleavage site between the HA1

TABLE 1
Sequence Comparisons of A/Goose/Guangdong/1/96 with Its Closest Genetic Relatives^a

Gene	Region of the gene compared	Virus with highest % identity with A/Goose/Guangdong/1/96	
		Nucleotide	Amino acid
PB2	28–2304	Ruddy Turnstone/47/85 (91.1%)	Ruddy Turnstone/47/85 (91.1%)
PB1	25–2301	Hong Kong/156/97 (93.6%)	Tk/Minnesota/833/80 (98.02%)
PA	25–2172	SW/Hong Kong/126/82 (92.7%)	Tk/Minnesota/833/80 (98.0%)
HA	21–1743	Hong Kong/156/97 (98.8%)	Hong Kong/156/97 (98.9%)
NP	21–1542	Equine/Jilin/1/89 (94.6%)	DK/New Zealand/76 (99.5%)
NA	29–1391	Parrot/Ulster/73 (90.4%)	Parrot/Ulster/73 (96.1%)
M	26–1007	DK/Hong Kong/193/77 (95.6%)	DK/Hong Kong/193/77 (98.8%)
NS	27–864	TK/England/50-92/91 (97.8%)	TK/England/50-92/91 (97.4%)

^a Nucleotide and amino acid sequences were compared, and identity was determined by FASTA (Wisconsin Package, version 9.0) searches of GenBank and European Molecular Biology Laboratory databases. The nucleotide sequences have been deposited in GenBank; Accession numbers are from AF144300 to AF144307.

and HA2 domains of HA is seen in highly pathogenic avian influenza viruses of the H5 subtype (7). Phylogenetic analysis of the HA1 domain of the HA gene revealed that the HA gene of the influenza A/Goose/Guangdong/1/96 virus clusters with those of the H5N1 viruses isolated in Hong Kong in 1997 (Fig. 1). The deduced amino acid sequence of the HA gene of A/Goose/Guangdong/1/96 was most closely related to that of the A/Hong Kong/156/97 (H5N1) virus (Table 1), from which it differed by seven amino acids (A/Goose/Guangdong/96 vs A/Hong Kong/156/97: 35 K to R, 94 D to N, 108 T to I, 138 H to L, 263 A to T, 387 D to N, and 413 Q to K). Among these amino acid differences, the change at amino acid residue 138 may be of antigenic and biological importance because it is located in antigenic site B, on the right edge of the receptor binding site defined previously for H3 subtype HA (15). The seven potential glycosylation sites reported in the HA of the A/Hong Kong/156/97 (H5N1) virus (4) were conserved in the HA of the A/Goose/Guangdong/1/96 virus. The sequences of the HA genes of the other 15 human influenza A (H5N1) viruses differed from that of the A/Hong Kong/156/97 virus by two to eight amino acid residues (4). The genetic similarity of the HAs of the A/Goose/Guangdong/1/96 and A/Hong Kong/156/97 viruses was therefore within the range seen among H5N1 viruses isolated during the Hong Kong outbreak. These data revealed that the HA gene of the A/Goose/Guangdong/1/96 virus is similar to those of the H5N1 viruses isolated in Hong Kong and indicate that the HA of the A/Goose/Guangdong/1/96 virus and the Hong Kong H5N1 viruses are derived from a common ancestor.

Phylogenetic analyses of the coding region of RNA segment six showed that the neuraminidase (NA) gene of the A/Goose/Guangdong/1/96 virus was related to avian N1 NA genes, which lie in a separate branch of the tree from the NA genes of human H1N1 viruses (Fig. 2). Nucleotide sequence comparisons revealed that the NA

gene of the A/Goose/Guangdong/1/96 virus was most closely related to that of the A/Parrot/Ulster/73 (H7N1) virus (Table 1); amino acid sequence comparisons revealed that the NA sequence of the A/Goose/Guangdong/1/96 virus differed from that of A/Parrot/Ulster/73 by 23 amino acids and from that of A/Hong Kong/156/97 by 27 amino acids. Amino acids predicted to define the sialic acid binding site based on that of the N2 NA (16) were conserved in A/Goose/Guangdong/1/96, with the exception of residue 121, which is Ile in all other reported N1 NAs. In addition, the 19-amino-acid deletion de-

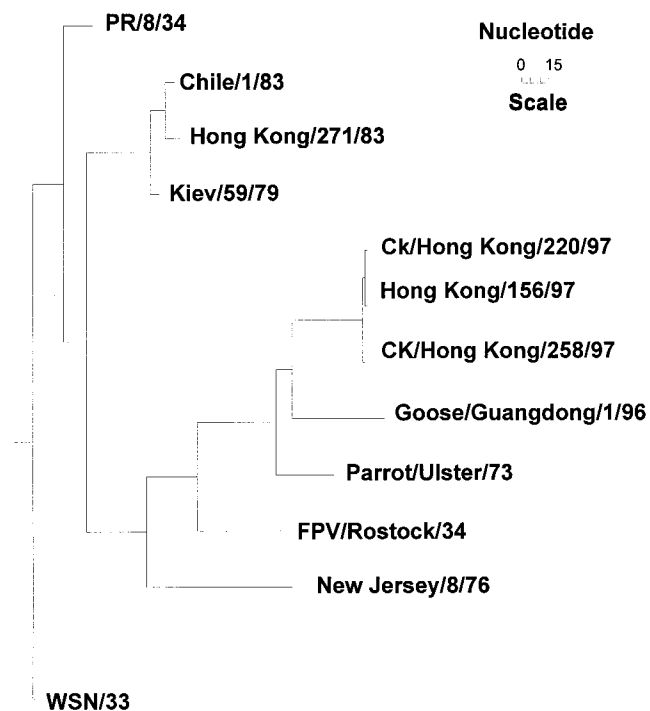


FIG. 2. Phylogenetic relationships of the neuraminidase gene of representative human and avian N1 subtype viruses and the A/Goose/Guangdong/1/96 virus. The tree is rooted to A/WSN/33 virus.

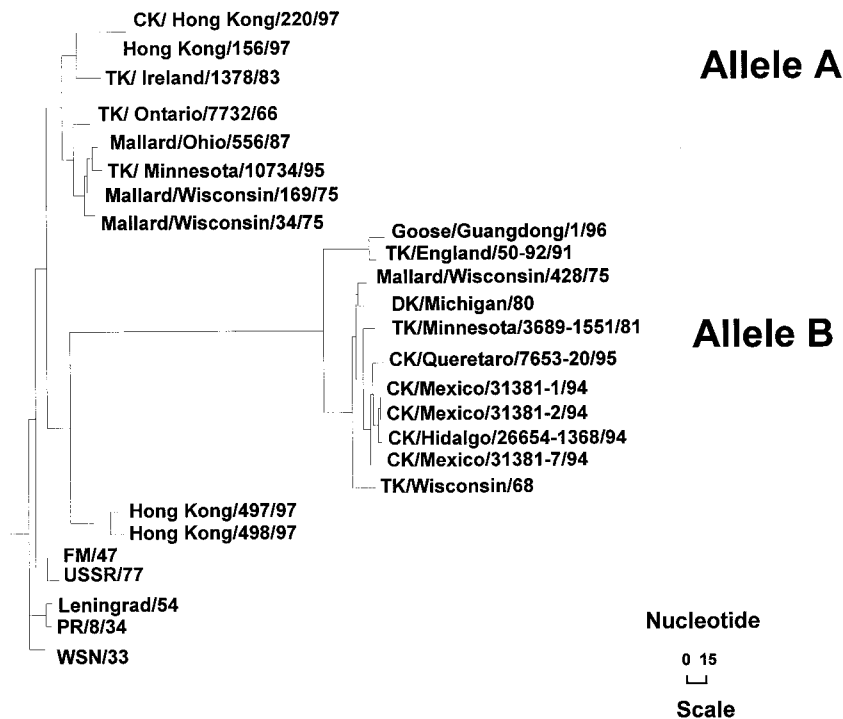


FIG. 3. Phylogenetic relationships of the NS gene of representative human and avian influenza A viruses and that of A/Goose/Guangdong/1/96 virus. The tree is rooted to the A/WSN/33 virus.

scribed in the stalk region of Hong Kong viruses (13, 14) was not seen in the NA of the A/Goose/Guangdong/1/96 virus; the latter, therefore, did have the three potential glycosylation sites in the stalk of the NA that were deleted in the Hong Kong viruses (4).

Influenza A viruses with the greatest nucleotide and amino acid sequence similarity to the polymerase (PB2, PB1, PA), NP, and M genes of A/Goose/Guangdong/1/96 virus are presented in Table 1. Amino acid similarities showed that each of these five genes of the A/Goose/Guangdong/1/96 virus was closely related to an avian influenza virus other than A/Hong Kong/156/97. Although the PB1 gene of A/Goose/Guangdong/1/96 was most closely related at nucleotide level (93.6%) to that of A/Hong Kong/156/97 (146 nucleotide and 30 amino acid differences between the two viruses), it was most closely related at the amino acid level to that of A/TK/Minnesota/833/80 (231 nucleotide and 16 amino acid differences between the two viruses). In addition, A/Goose/Guangdong/1/96 lacked the extra amino acid described in the 3' end of the PB1 protein of the H5N1 viruses isolated from Hong Kong (6). The PA and NP genes of A/Goose/Guangdong/1/96 were most closely related to A/Swine/Hong Kong/126/82 (H3N2) and A/Equine/Jilin/1/89 (H3N8) viruses (Table 1), respectively, based upon nucleotide similarity. These two viruses are believed to be avian viruses that crossed the host species barrier to infect pigs and horses, respectively (17, 18). Figure 3 shows the phylogenetic relationships of the NS gene. The phylogram readily identifies the two previously de-

scribed alleles of the NS gene (19). The NS gene of the A/Goose/Guangdong/1/96 virus belongs to allele B and is most closely related to that of A/Turkey/England/91 (H5N1) on the basis of both nucleotide and amino acid similarities (Table 1). In contrast, the NS gene of H5N1 viruses isolated from Hong Kong belongs to allele A (Fig. 3).

Thus, the NA and internal genes of the A/Goose/Guangdong/1/96 virus have greater sequence similarities to other avian influenza viruses than to the H5N1 viruses isolated in Hong Kong (Table 1). These results suggest that the A/Goose/Guangdong/1/96 and Hong Kong H5N1 viruses derived these seven gene segments from one or more different progenitors circulating among avian species.

Cocirculation of multiple subtypes of influenza viruses allows opportunities for genetic reassortment to occur. Shortridge reported that during surveillance conducted between 1975 and 1980, at least 10 subtypes of influenza A viruses were isolated from domestic ducks, geese, and chickens in Hong Kong and southern China, although most of these viruses were apathogenic (20). The frequency with which different HA subtypes were detected among the duck isolates was H4 (29%) > H3 (25%) > H6 (22%) > H10 > H5 > H9 > H11 > H2 > H1 > H7 (20). In addition to the highly pathogenic H5N1 virus outbreak in geese in Guangdong Province, H9N2 viruses which caused illness with low mortality were isolated from sick chickens on farms in Sichuan and Xinjiang Provinces (12). Cocirculation of multiple subtypes of influenza A

viruses in this region suggests that surveillance for influenza in humans as well as in avian and swine populations must be maintained.

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